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Amplification of the *c-MYC* oncogene has been found in five to twenty percent of breast tumors. However, the molecular mechanisms by which *c-MYC* contributes to tumorigenesis is not well understood. To address this question, we have used the tetracycline regulatory system to conditionally express the *c-MYC* oncogene in the mammary epithelium of bitransgenic mice. To determine whether c-MYC induced mammary tumors remain dependent on c-MYC for maintainenance of the tumorigenic state, we deinduced *c-MYC* expression in animals harboring tumors. Withdrawal of *c-MYC* expression revealed that nearly half of the tumors remain dependent on *c-MYC* transgene expression for their continued growth.

To study the molecular properties that distinguish c-MYC dependent and independent tumors, c-MYC initiated tumors were analyzed for mutations in the *ras* family of genes. Approximately half of the tumors examined were found to harbor spontaneous activating point mutations in either *K-ras* or *N-ras*, whereas no mutations were found in *H-ras*. Interestingly, the presence of an activating *ras* mutation was found to strongly correlate with the failure of tumors to regress following c-MYC deinduction. These data suggest that spontaneous activating mutations in K-*ras* and N-*ras* represent a preferred secondary pathway for c-MYC-induced tumorigenesis in the mammary gland.

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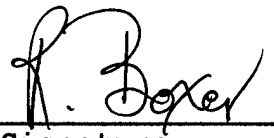

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12/19/01

To Whom It May Concern,

Please find attached my progress report for the period covering July 01, 2000 – June 30, 2001. Although I initially wrote my predoctoral traineeship award application for a project studying the role of murine BRCA1 protein interactions in DNA repair, by the time that the award was granted, I had determined that the proposed project was not going to be feasible to complete. Accordingly, I began working on another breast cancer related project that was ongoing in our lab. Our lab developed an inducible system to study the role of c-MYC expression in the mammary gland. My project particularly focuses on the selection of secondary oncogenic pathways during c-MYC initiated mammary gland tumorigenesis. Our studies with this model have been very productive and have already resulted in reportable outcomes (D'Cruz et al, Nature Medicine 7, 235-239, 2001). I have included in my progress report a description of our initial findings with this system as well as the new Specific Aims and Statement of Work for my project.

Sincerely,

Robert Boxer

Introduction:

The product of the c-MYC gene has been implicated in human breast cancer for many years. The c-MYC oncogene is amplified in five to twenty percent of human breast cancers and this amplification is associated with aggressive tumor behavior and poor prognosis. However, the exact molecular mechanisms through which c-MYC contributes to breast cancer are largely unknown. Although the process of mammary tumorigenesis requires multiple genetic events, it is unclear to what extent c-MYC initiated carcinogenesis proceeds through preferred secondary pathways. In addition, the extent to which established mammary tumors remain dependent on individual mutations for maintenance of the transformed state is also unknown. To address these questions, our lab has used the tetracycline regulatory system to conditionally express the human c-MYC oncogene in the mammary epithelium of transgenic mice.

Our lab has previously reported the generation of bitransgenic mice in which the reverse tetracycline-dependent transcriptional activator (rtTA) under the control of the mouse mammary tumor virus long terminal repeat directs the conditional expression of c-MYC in the mammary gland, referred to as MTB/TOM mice. Chronic administration of doxycycline to MTB/TOM female mice resulted in the formation of mammary gland tumors with high penetrance and an average latency of 22 weeks. The long latency period and stochastic formation of tumors in MTB/TOM mice indicated that additional mutations were likely to be required for tumorigenesis. As forced co-overexpression of Hras1 and Myc in the mammary glands of transgenic mice has been shown to accelerate mammary tumorigenesis, c-MYC-induced mammary tumors were examined for spontaneous activating mutations in H-, K-, and N-ras. Although none of the tumors were found to harbor mutations in H-ras, about half of the tumors were found to have spontaneous activating point mutations in either K-ras or N-ras, with K-ras mutations being three times more likely than N-ras mutations. These results suggest that deregulated expression of c-MYC in the mammary gland strongly selects for spontaneous activating mutations in ras family members as a preferred secondary oncogenic pathway.

To determine whether c-MYC overexpression is required for the maintenance of mammary adenocarcinomas, doxycyline was withdrawn from chronically induced, tumor-bearing MTB/TOM mice. In approximately half of the tumors, doxycycline withdrawal resulted in the rapid regression and clinical disappearance of invasive mammary adenocarcinomas, indicating that maintenance of these tumors is dependent on c-MYC transgene overexpression. In contrast, the other half of the tumors either continued to grow following doxycycline withdrawal or decreased in size to a variable extent and then resumed growth. In this report, we describe our ongoing experiments in which we address the role of ras activation in c-MYC induced mammary tumorigenesis. Specifically, these experiments seek to determine whether the presence of a ras mutation allows for the c-MYC independent growth of mammary gland tumors initiated by c-MYC overexpression.

Specific Aims:

(I) Determine whether the presence of a ras mutation correlates with the inability of a tumor to fully regress following doxycycline withdrawal.
(II) Determine the molecular differences between MTB/TOM tumors with K-ras mutations, N-ras mutations, and those without any detectable Ras mutations.
(III) Determine whether the presence of a K-ras mutation is necessary and/or sufficient to prevent tumor regression

Statement of Work:

Aim I: Determine whether the presence of a ras mutation correlates with the inhibition of tumor regression

Task 1: Months 1-4: Generate MTB/TOM bitransgenic animals and induce animals with Doxycycline.
Task 2: Months 4-7: Monitor animals for tumors
Task 3: Months 4-7: Biopsy tumors, deinduce animals, and monitor tumors for regression.
Task 4: Months 7-9: Prepare RNA from frozen biopsy tissue samples.
Task 5: Months 9-12: PCR amplify, purify, and sequence fragments from H, K, and N-ras transcripts for mutations at codons 12, 13, and 61.

Aim II: Determine the molecular differences between MTB/TOM tumors with K-ras mutations, N-ras mutations, and those without any detectable Ras mutations.

Task 1: Months 1-12: Generate cohorts of MTB/TOM MG tumors to use for comparison of gene expression between tumors with K-ras mutations, N-ras mutations, and those without any detectable Ras mutations.
Task 2: Month 13: Generate biotinylated cRNA from RNA prepared from tumor cohorts.
Task 3: Months 14-15: Run biotinylated cRNA on Affymetrix Microarrays.
Task 4: Months 16-18: Use bioinformatic programs to analyze microarray data for statistically significant differences in gene expression.
Task 5: Months 19-21: Confirm differences in gene expression by Northern blot analysis of an independent set of tumors.
Task 6: Months 22-25: Confirm differences in gene expression at the protein level by Western blot analysis.
Task 7: Months 26-28: Examine the gene expression of N-ras mutant tumors for genes identified as having different expression in K-ras mutant tumors and tumors without any detectable Ras mutations.

Aim III: Test whether K-ras is necessary and/or sufficient to inhibit tumor regression.

Task 1: Months 27-29: Construct retroviral vectors for the expression of an activated K-ras and of a K-ras antisense sequence.
Task 2: Months 29-30: Generate high titre retroviruses to be used for infections.
Task 3: Months 31-32: Infect MTB/TOM cells in culture.
Task 4: Months 33-34: Inject cells into the flanks of recipient FVB mice to generate infected tumor grafts.
Task 5: Months 35-36: Biopsy and deinduce grafts. Monitor grafts for regression.

Progress:

Aim I: Determine whether the presence of a ras mutation correlates with the inhibition of tumor regression

Tasks 1-3 (Months 1-7): Generation of a cohort of biopsied MTB/TOM Mammary Gland Tumors with Regression Information

In order to determine whether the presence of a ras mutation correlates with the inability of tumors to fully regress following the deinduction of c-MYC transgene expression, bitransgenic MTB/TOM animals were generated for the induction of mammary gland tumors. Accordingly, the transgenic mouse line expressing the reverse tetracycline-dependent transcriptional activator (rtTA) under the control of the mouse mammary tumor virus long terminal repeat (mouse line designated here MTB) were mated to a second transgenic line containing exons 2 and 3 of human MYC under the control of the tetracycline-dependent minimal promoter (mouse line designated here TOM). At six weeks of age, bitransgenic female progeny from this cross were administered 2.0 mg/mL Doxycycline and 5% sucrose in their drinking water to induce the c-MYC transgene chronically for tumor initiation. Sixty-nine mammary gland tumors arising in MTB/TOM bitransgenic mice were biopsied. Following a week recovery period, doxycycline was removed from the drinking water of biopsied animals to deinduce the c-MYC transgene. Deinduced tumors were then measured approximately every four days to determine the extent of tumor regression. Of the sixty-nine tumors, forty percent (28/69) fully regressed to a clinically undetectable state.

Tasks 4 and 5 (Months 7-12): Detection of Ras mutations in MTB/TOM Mammary Gland Tumors with Regression Information

In order to determine whether the inability of tumors to fully regress following c-MYC transgene deinduction correlates with the presence of a ras mutation, tissue samples acquired from the biopsy were examined for the presence of activating point mutations in ras family members. Accordingly, cDNA prepared from frozen biopsy tissue samples were used to PCR amplify fragments of the H-ras, K-ras, and N-ras genes. These fragments were then sequenced to determine the presence of activating point mutations at codons 12, 13, and 61. Twenty-six of the sixty-nine tumors possessed activating point mutations in K-ras, while an additional nine tumors had activating point mutations in N-ras. The remaining thirty-four tumors did not have any detectable activating point mutations in H-ras, K-ras, or N-ras.

Combining the information acquired about tumor regression in Task 3 with the information about the presence of a ras mutation, we determined that the presence of a K-ras mutation correlates with the inhibition of tumor regression. The vast majority of tumors bearing a K-ras mutation did not fully regress (24/26) following deinduction of the c-MYC transgene. In contrast, only forty percent of tumors without detectable mutations in H-ras, K-ras, or N-ras incompletely regressed (14/35), whereas the remaining sixty percent of tumors fully regressed beyond clinical detection. Surprisingly,

the presence of an activating N-ras mutation did not correlate with the inability of the tumor to fully regress. Similar to tumors without a detectable ras mutation, nearly sixty percent of tumors with an activating N-ras mutation fully regressed to an undetectable state (5/9). These results are surprising considering the high degree of homology between K-ras and N-ras and suggest that K-ras and N-ras may have distinct activities in the mammary gland.

Aim II: Determine the molecular differences between MTB/TOM tumors with K-ras mutations, N-ras mutations, and those without any detectable Ras mutations.

Task 1 (Months 1-12): Generate cohorts of MTB/TOM MG tumors to use for comparison of gene expression between tumors with K-ras mutations, N-ras mutations, and those without any detectable Ras mutations.

As described in Aim I, MTB/TOM bitransgenic animals were chronically induced with doxycycline. Tumors arising in these animals were biopsied and tissue samples were frozen for molecular analysis. Frozen biopsy tissue samples were used to generate RNA. The tumor RNA was then used to produce cDNA for the PCR amplification of H-ras, K-ras, and N-ras genes. The amplified fragments were then sequenced to determine the presence of activating point mutations at codons 12, 13, and 61 in the ras genes. As described above, twenty-six of the sixty-nine tumors possessed activating point mutations in K-ras, while an additional nine tumors had activating point mutations in N-ras. The remaining thirty-four tumors did not have any detectable activating point mutations in H-ras, K-ras, or N-ras. Selected tumors from these cohorts will be used for a comparison of gene expression in an Affymetrix Microarray analysis.

Tasks 2-7 (Months 13-28):

In accordance with our statement of work, these studies will be initiated in the near future.

Aim III (Months 27-36): Test whether K-ras is necessary and/or sufficient to inhibit tumor regression.

In accordance with our statement of work, these studies will be initiated in the near future.

Key Research Accomplishments:

-Determined that the presence of a spontaneous activating point mutation in K-ras correlates with the inability of a c-MYC initiated mammary gland tumor to fully regress in the absence of c-MYC expression.

Reportable Outcomes:

c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous *Kras2* mutations. D'Cruz et al., Nature Medicine, 7: 235-239, 2001.

Conclusions:

The identification of c-MYC gene amplification in five to twenty percent of human breast cancers suggests that the overexpression of the c-MYC gene product contributes to mammary gland tumorigenesis. This idea is further supported by expression analysis of human breast cancers, which demonstrates increased c-MYC expression levels in approximately seventy percent of the examined tumors. Despite this, the molecular mechanisms by which c-MYC overexpression contributes to mammary gland tumorigenesis remain to be fully elucidated. In order to help discern the role of c-MYC in mammary gland tumorigenesis, our lab has created a bitransgenic mouse line in which c-MYC can be inducibly expressed in the mammary gland. This animal model has been used by our lab to answer the questions of whether c-MYC initiated mammary gland tumors remain dependent upon c-MYC expression for their continued growth and whether c-MYC initiated tumors select for mutations in preferred secondary oncogenic pathways. Our initial studies indicate that approximately fifty percent of c-MYC initiated mammary gland tumors remain dependent upon c-MYC for their continued growth. We have also determined that nearly fifty percent of the tumors select for activating point mutations in ras genes with a strong preference for K-ras over N-ras, and both K-ras and N-ras over H-ras. As part of the current proposal we have found that the presence of a K-ras mutation significantly correlates with the inability of the tumor to fully regress following deinduction of the c-MYC transgene. However, the presence of an N-ras mutation does not correlate with the inability of the tumor to fully regress. Accordingly, N-ras mutant tumors behave more similarly to tumors without any detectable ras mutations than tumors bearing K-ras mutations. These results are surprising considering the high degree of homology between K-ras and N-ras and suggest that K-ras and N-ras may have distinct activities in the mammary gland. Our ongoing studies will examine the differences in gene expression between MTB/TOM tumors with and without ras mutations. In addition, we seek to determine whether the presence of a K-ras mutation plays a causal role in the inhibition of tumor regression following c-MYC transgene deinduction. We believe that our studies by identifying specific sets of preferentially associated oncogenic mutations will facilitate targeting of the multiple synergistic pathways that contribute to neoplastic growth.

References:

D'Cruz et al, Nature Medicine 7, 235-239, 2001. c-MYC induces mammary tumorigenesis by means of a preferred pathway involving spontaneous *Kras2* mutations.

Escot, C., et al. Proc. Natl. Acad. Sci. USA. 83: 4834-4838, 1986. Genetic alteration of the c-myc protooncogene (*MYC*) in human primary breast carcinomas.